The impact of the Amphibian Chytrid Fungus *Batrachochytrium dendrobatidis* on a Green and Golden Bell Frog *Litoria aurea* reintroduction program at the Hunter Wetlands Centre Australia in the Hunter Region of NSW

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ABSTRACT

Reintroduction programs are used widely in conservation to reduce a species' risk of extinction and amphibians are considered suitable candidates for such programs because of their behavioural simplicity and high reproductive output. The Green and Golden Bell Frog *Litoria aurea* is an endangered species that has been reintroduced into several areas within its natural range, but the outcome of these programs remain unknown. This paper presents the results from the first release of the bell frog in the Hunter Region of New South Wales. This reintroduction released 850 tadpoles into a closed system of three ponds and rehabilitated habitat. Tadpole survival was high but following metamorphosis a decline in numbers began that continued for 13 months and resulted in the disappearance of all released bell frogs. The cause of this decline was investigated and eventually attributed to infection by the Amphibian Chytrid Fungus *Batrachochytrium dendrobatidis*. These results emphasize the importance of including regular chytrid testing in the monitoring of both natural populations and reintroduction programs, particularly as few sick and dead animals were found to indicate its presence.

Key words: *Litoria aurea,* green and golden bell frog, reintroduction, translocation, amphibian chytrid fungus, *Batrachochytrium dendrobatidis,* chytridiomycosis.

Introduction

Reintroduction programs are used in conservation to reduce a species' risk of extinction by establishing new populations in areas that they have declined or disappeared from (Jungius 1985; IUCN 1987; Dodd and Seigel 1991; Serena and Williams 1995; Seddon *et al* 2007). They also raise the perceived value of land, increase public awareness and participation in conservation efforts and provide information on the population dynamics, ecology and management of the study species (Dodd and Seigel 1991; Serena and Williams 1995). Reintroduction programs require extensive planning, research and years of monitoring and should result in a self sustaining population that interacts freely with its environment and requires minimal management (Dodd and Seigel 1991; Serena and Williams 1995). However, reintroduction programs are rarely successful in achieving these outcomes and their value in conservation is constantly questioned (Jungius 1985; Conant 1988; Wirth 1990; Lindburg 1992). Despite this, reintroduction programs are widely implemented (Jungius 1985; Griffith *et al* 1989; Dodd and Seigel 1991; Seddon *et al* 2007) particularly for amphibians (Bloxam and Tonge 1995).

Amphibians are suitable candidates for reintroduction programs as they are behaviorally simple, requiring no pre-release training and have high reproductive outputs, allowing them to establish large populations within several generations (Bloxam and Tonge 1995). The Green and Golden Bell Frog *Litoria aurea* is an endangered species that has been declining for the last 30 years. The cause of this decline has not been determined although habitat modification, predation by the introduced fish *Gambusia holbrooki* and the Amphibian Chytrid Fungus *Batrachochytrium dendrobatidis* have been implicated (Pyke and White 2001; Pyke *et al* 2002; DEC NSW 2005). As such, the introduction of the bell frog into rehabilitated or supplementary habitat has been widely used in its conservation with programs currently occurring in Botany, Marrickville, Collaroy, Arncliffe and Pambula (DEC NSW 2005; Daly *et al* 2008; Pyke *et al* 2008) .

Despite the number of bell frog reintroduction programs underway, their design, methodology and progress remain unknown because records have rarely been published in peer-reviewed journals. This paper describes the outcome of the first release of the green and golden bell frog in a reintroduction program occurring in the Hunter Region of New South Wales (NSW). The aim of this release was to establish the first generation of bell frogs on site in the absence of the threatening processes considered to be responsible for its decline.

Methods

Study Site

Hunter Wetlands Centre Australia owns and manages the Shortland Wetlands in the Hunter Region of NSW (32°52'19"S 151°42'90"E) including 45 hectares of rehabilitated wetlands and a visitor centre. It is currently listed as a Ramsar wetland of international importance and functions as a community based environmental, educational and ecotourism facility. Bell frogs were once widespread throughout Shortland but became locally extinct in the 1970s when the wetlands were filled for development. In May 2002 construction of ponds began in a secluded area of overgrown grasslands following the specifications of Pyke and White (1996). Three ponds of varying sizes were excavated (dimensions 15.7 x 12.4 m, 14.5 x 11.6 m and 12.8 x 4.9 m) and vegetated with the emergent Cumbungi *Typha orientalis*. Garden beds containing locally endemic grass and shrub species were placed around and between each pond and six rock piles were also placed either between or extending into each pond (Fig 1, 2).

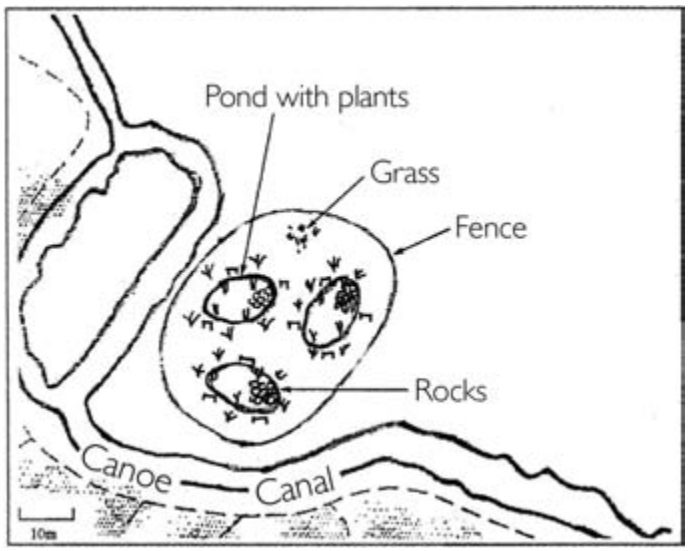

Figure 1. Site map of the Hunter Wetlands Centre Australia Green and Golden Bell Frog reintroduction site in the Hunter Region of NSW (source: Jamieson and Mahony 2003).

A 1 m tall mesh fence was placed around the reintroduction site, containing the ponds and 2700m^2 of surrounding grassland (Fig 2). This fence was buried 20 cm into the ground, had 20 cm overhangs at the top on both sides and was designed to keep bell frogs inside the enclosure so population numbers could be monitored closely. It was also to keep other species out to prevent interspecies competition, predation and to reduce the chances of chytrid being carried into the site. However, prior to the construction of this fence one species of ground frog, the Common Eastern Froglet *Crinia signifera* became established within the site despite efforts to remove it. Six of these froglets were sent to the CSIRO Australian Animal Health Laboratory for chytrid testing and all came back negative.

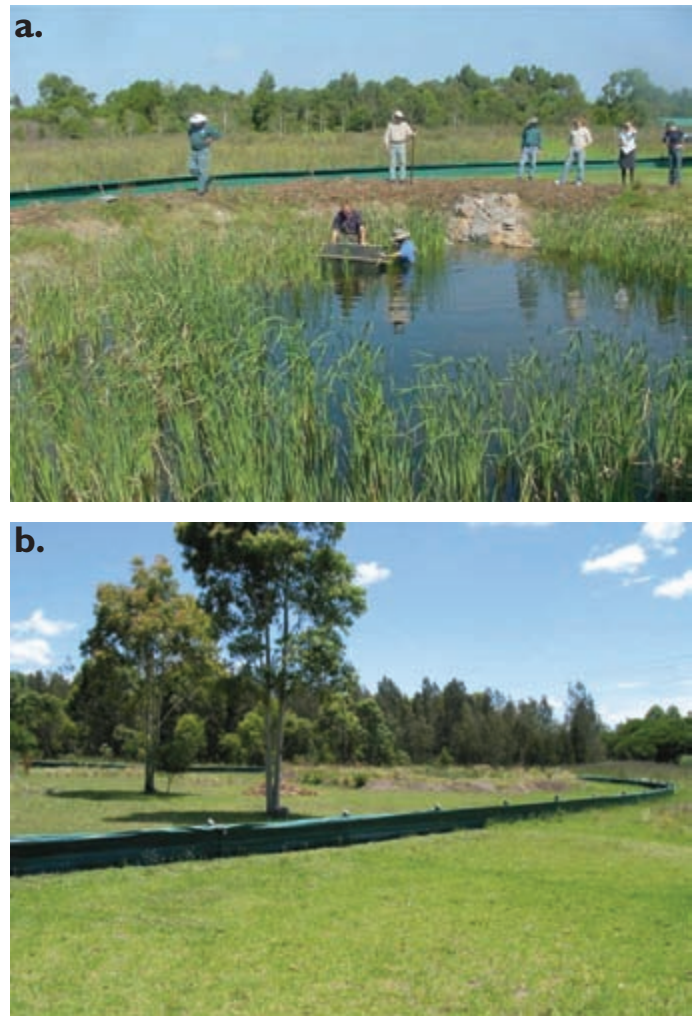

Figure 2. The Hunter Wetlands Centre Australia Green and Golden Bell Frog reintroduction site showing: (a) one of the three ponds with emergent Cumbungi vegetation, a rock pile extending into the water and a mesh holding basket being placed into the pond and, (b) the 1 m tall fence surrounding the reintroduction site.

Ponds were filled with fish-free groundwater, pumped from a nearby creek with hoses covered in 2 mm mesh to exclude any fish. Prior to the release of bell frog tadpoles into the reintroduction site the ponds were left to stand for 12 months. During this time two species of tree frog, the Dwarf Green Treefrog *Litoria fallax* and the Emerald Spotted Treefrog *Litoria peronii*, breached the fence and also became established within the enclosure.

Release and Survey

Bell frog tadpoles were initially intended to be bred from wild animals from the Sandgate population, less than 3 km away (32°53'00"S 151°42'05"E) from the reintroduction site. However, the source population appears to have undergone a decline and possible localised extinction (M. Mahony pers. obs.) so source animals could not be found. Tadpoles were instead bred from 2 captive animals on display at the Hunter Wetlands Centre Australia that were initially from the Sandgate population.

In the summer of 2004, 20 bell frog tadpoles were bred at the University of Newcastle and released into a mesh

basket (dimensions 1 m^3) submerged in one of the ponds at the Hunter Wetlands Centre Australia reintroduction site (Fig 2). This release was intended to assess whether the habitat was likely to be suitable for the growth and survival of bell frogs. Eleven of these tadpoles metamorphosed and were collected from the basket and released freely into the pond. The remaining 8 tadpoles disappeared from the basket and may have either died and decomposed or metamorphosed and climbed out. At least 3 of these metamorphs survived for more than two years within the reintroduction site. This confirmed that the enclosure was likely to be suitable and the reintroduction program continued.

In the summer of 2005, 850 bell frog tadpoles were bred at the University of Newcastle and grown to Gosner stages 35-38 (Gosner 1960) to prevent the loss of animals to small invertebrate predators (Pyke and White 2001). Prior to release the mouthparts of 60 randomly selected tadpoles were checked under a stereomicroscope for signs of pigment loss associated with chytrid infection (Fellers *et al* 2001; Marantelli *et al* 2004; Rachowicz and Vredenburg 2004). No pigment loss was found in any of the animals tested so the release continued. The sample size of 60 animals was chosen throughout this study for the monitoring of chytrid infection because it allows the detection of one positive animal with a likelihood of 95%, if the prevalence within the population is 5% or greater (DiGiacomo and Koepsell 1986).

Upon release 650 of the tadpoles were placed freely into the ponds and 200 were distributed into four mesh baskets partially submerged into each of the ponds. The tadpoles in the baskets were easily accessible and could be monitored closely to ensure that the water quality of the ponds was suitable for their growth and survival. The tadpoles in the mesh baskets were counted daily until metamorphosis when they were weighed and released freely into the ponds. The free-living metamorph numbers were then monitored over time using time-constrained standardised visual encounter surveys (Crump and Scott 1994) at a frequency of 2-4 times per month. Species-time curves were generated to determine the minimal search time in each pond that detected the maximum number of frogs visible and it was found that 20 minutes in the two larger ponds and 10 minutes in the smaller pond were appropriate. These surveys were standardised by using the same investigator and following the same paths within the ponds each time. The number of bell frogs, dwarf treefrogs and spotted treefrogs seen in each survey were recorded. Due to the cryptic nature of the eastern froglets, their numbers were estimated based on the number heard calling at the beginning of each survey.

At each survey event the ponds were also monitored for predators and water depth was measured using permanent depth markers placed at the deepest point in each pond. The pH of the water in each pond was measured using a Hanna Instruments pHep meter while the dissolved oxygen and salinity were measured using a YSI Model 85 water test kit. Opportunistic night surveys were also conducted during rain events around near-by water bodies to determine whether bell frogs were able to disperse out of the enclosure. Any dead animals found were swabbed with sterile fine-tip MW-100 swabs and stored in 80% ethanol.

On individual nights in June and December a sample of bell frogs were located with spotlight searches and caught by hand, where the hand was covered in a single-use plastic bag to prevent the spread of disease. Once an animal was caught the bag was inverted to contain the animal where it was weighed on digital scales and swabbed for chytrid infection by wiping the swab over the abdomen, inner thigh, hands and feet of each animal in a standardised manner. The DNA on these swabs was extracted at The University of Newcastle and DNA from the chytrid fungus was amplified using real-time Taqman PCR assays, following the methods described by Boyle *et al.* (2004). This test quantifies the number of chytrid zoospore equivalents present and is then multiplied by 10 to account for a 1/10 dilution step in the extraction process. This zoospore count provides a measure of infection severity that is comparable between animals.

In order to prevent the spread of disease in and out of the site all equipment and footwear of investigators and grounds keepers were washed in hot tap water (~50 °C) and sprayed with a 2% sodium hypochlorite solution prior to entry, both of which will kill chytrid zoosporangia within 30 seconds (Johnson *et al* 2003) and are recommended in hygiene protocols (NPWS 2000). Wader use was restricted to this site only.

Results

In the 2005 release 73% of tadpoles placed into 3 of the mesh baskets survived and metamorphosed with an average weight (± SD) of 1.09 g (± 0.34) by the end of March 2005. The tadpoles placed into the fourth mesh basket escaped when the lid floated open during a heavy rain event. The visual encounter survey of free-living bell frogs also found metamorphs emerging throughout March and individuals were observed to survive for up to a year in this enclosure although numbers decreased throughout the year (Figure 3). A gradual decline occurred from May 2005 and continued until the end of August. The rate of decline then appeared to slow until November when it increased again (Figure 3). No bell frogs have been seen in the enclosure since the end of March 2006. Six frogs (only bell frogs) were found dead within the enclosure between June and August 2005. Only five of these animals were in an early enough state of decay to swab and four were found to be infected with chytrid. The number of zoospores from each of these was highly variable with an average (± SD) of 3489° (± 6455°) spores. None of these frogs showed any skin lesions, excess shed skin or ventral reddening.

On one night in June, 60 bell frog juveniles were caught and found to have an average weight (± SD) of 3.76 g (± 1.27). These 60 animals were swabbed for chytrid and analysis revealed it was present on 53% of individuals. The number of zoospores from each sample was highly variable with an average (± SD) of 50 (± 124) spores. The December night survey took place but was only able

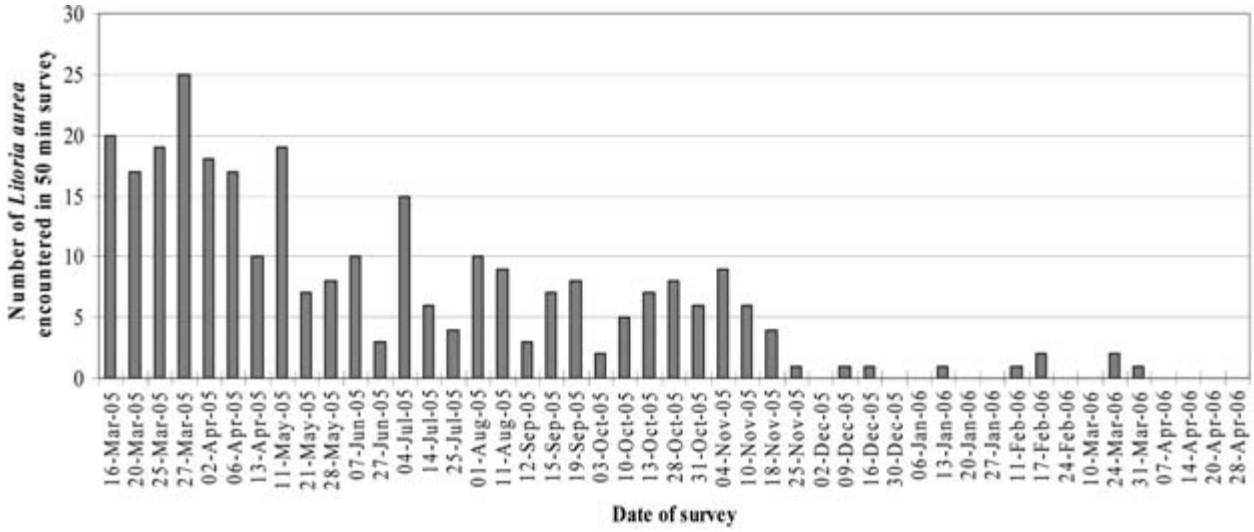

Figure 3. The number of Green and Golden Bell Frogs encountered in each survey over the first year following release at the Hunter Wetlands Centre.

to locate 4 bell frogs, none of which could be caught so growth and infection data could not be collected. However, in the absence of bell frogs, 60 dwarf treefrogs, 10 eastern froglets and 8 spotted treefrogs were caught and swabbed. Of these animals 2 dwarf treefrogs tested positive for chytrid with an average (± SD) zoospore count of 1.8 (± 0.3). All other animals were negative for the presence of chytrid.

The number of dwarf treefrogs andspotted treefrogs encountered during surveys did not decline in parallel with bell frogs (Figure 4). Both species were observed less frequently and in lower numbers from May to August but reappeared in October. Dwarf treefrogs were observed in almost three times the numbers of bell frogs and peaked in number during December 2005 and January 2006. spotted treefrog numbers remained consistently low throughout the study (Fig. 4). The number of calling eastern froglets at the start of each survey also did not decline in parallel with the bell frogs (Fig. 5). It was heard at a lesser frequency and in lower numbers from September 2005 to February 2006, but increased after this period.

During these visual encounter and night surveys bell frogs were often observed making use of the constructed habitat, basking and foraging on emergent and surrounding vegetation and hiding in rock piles within the enclosure. The range of depth and water quality values was found to be similar within the three ponds (Table 1). Several frog predators were observed within the enclosure during surveys including the White Faced Heron *Egretta novaehollandiae*, the Australian Raven *Corvus coronoides*, the Pied Butcherbird *Cracticus nigrogularis* and the Eastern Water Skink *Eulamprus quoyii.* The scats of a large rodent and the tracks of a fox *Vulpes vulpes* have also been found within the enclosure. Each of these predators was observed on fewer than 3 occasions each. The Mosquito Fish *Gambusia holbrok* predator was never observed in the reintroduction site.

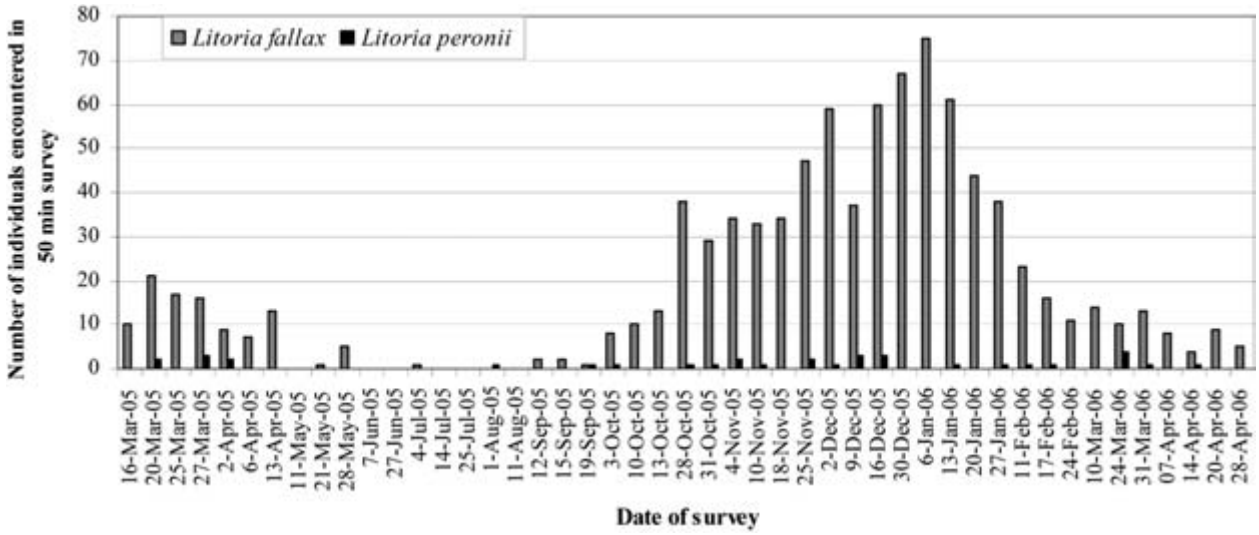

Figure 4. The number of Dwarf Green Treefrogs *Litoria fallax* and Emerald Spotted Treefrogs *Litoria peronii* encountered in each survey over the first year at the Hunter Wetlands Centre bell frog reintroduction site.

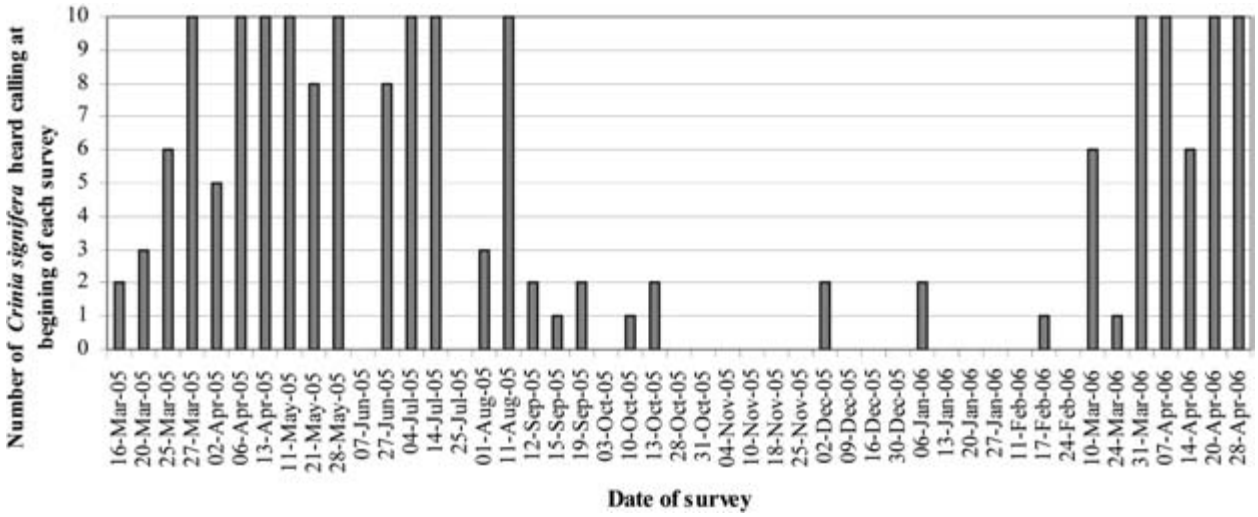

Figure 5. The number of Common Eastern Froglets *Crinia signifera* heard calling at the beginning of each survey over the first year at the Hunter Wetlands Centre bell frog reintroduction site.

Table 1. Range of depth, pH, dissolved oxygen, and salinity of the three ponds at the Hunter Wetlands Centre bell frog reintroduction site.

	Depth	pH	Diss. O_2 (mg/L)	Salinity (ppt)
Pond 1	69-118	6.9-8.1	9.04-11.66	0.8-1.2
Pond 2	86-118	6.8-8	7.61-14.05	0.1-1.1
Pond 3	69-120	6.8-7.9	7.37-10.86	0.1-0.5

Discussion

This paper describes the outcome of the first release of bell frogs at the Hunter Wetlands Centre reintroduction site in the Hunter Region of NSW. A total of 850 bell frog tadpoles were released into three ponds and monitored 2-4 times a month. Two months after metamorphosis the number of bell frogs encountered during visual surveys began to decline (Figure 3). This decline continued throughout the year and within 13 months of the release, bell frogs appeared to have disappeared from the site. There is a possibility that a small number of animals persist but have not been observed and continued monitoring will determine this. Despite this possibility it is unlikely that this release will achieve its aim of establishing the first generation of bell frogs at this site. However, it has provided an opportunity to investigate why the bell frog was unable to persist and to improve the success of future reintroductions.

There are a number of reasons why observations of individuals in a population may decline. These include decreased visibility, dispersal and death. An observed decline may occur during visual encounter surveys if a change in the environment, such as increased vegetation growth or increased habitat complexity reduced the animal's visibility. Should such an increase occur gradually over time it could give the appearance of a decline. However, no environmental change was observed at the reintroduction site. In addition, such change would reduce the visibility of all species and as the pattern of fluctuations in the number of bell frogs differed from that of dwarf treefrogs, spotted treefrogs and eastern froglets it is unlikely to have been the cause of the observed decline. A change in the animal's behaviour like that of over-winter sheltering could also reduce the animal's visibility and explain an observed decline. Bell frogs generally become less visible during winter as they retreat to shelter sites such as thick vegetation, mud and rock piles (Pyke and White 2001) that have higher temperatures than surrounding areas, allowing them to sustain higher body temperatures (Duellman and Trueb 1994; Pyke and White 2001; Hamer *et al* 2003). This behaviour could explain the initial winter decline in bell frog numbers in this study, but an increase in spring would be expected. This increase in numbers with increasing temperature was observed for dwarf treefrogs and spotted treefrogs which also shelter overwinter (Fig. 4) but not for the bell frog (Fig. 3) making it unlikely that the observed decline was due to a change in the animal's behaviour.

The observed decline may also be the result of animals dispersing from the enclosure. Many amphibian species are known to disperse from their natal ponds as recently metamorphosed young and juveniles (Duellman and Trueb 1994). Bell frogs are known to move long distances as both juveniles (Goldingay and Newell 2005) and adults (Pyke and White 2001; Hamer 2002). The enclosure was fenced to prevent this dispersal but the ability of dwarf treefrogs and spotted treefrogs to climb this fence raises the possibility that bell frogs may also have done so. The surveys of surrounding water bodies outside of the enclosure found no sign of bell frogs making dispersal unlikely to be the cause of decline. However, this does not rule out the possibility that animals dispersed beyond these ponds.

The final possibility is that the reintroduced bell frog population was observed to decline because of mortalities. The suitability of habitat to meet all of a species' requirements is essential to the success of reintroduction programs (Dodd and Seigel 1991; Kleiman and Beck 1994; IUCN/SSC 1995). For this reason habitat conditions were monitored closely in this study. The metamorphosis of 73% of bell frog basket tadpoles is much higher than the survival rate normally observed for amphibians in nature (5-10 %, Duellman and Trueb 1994), indicating that the habitat was suitable for this species. The bell frog is tolerant of a wide range of conditions

and the range of water quality measurements for each pond (Table 1) was found to remain within the range observed in natural habitat (Pyke*et al* 2002) including nearby ponds on Kooragang Island (Hamer 2002). The use of habitat for foraging and basking by bell frogs in the reintroduction site, their high rate of growth from March to June during the initial decline and the survival and reproduction of the other species that often occur with bell frogs in nature also indicate that the habitat was suitable. Competition and predation are also processes that could result in a decline in a less competitive or prey species. However, none of the other species would be strong competitors of bell frogs at this site due to the low abundance of spotted treefrogs and the much smaller size of dwarf treefrogs and eastern froglets; nor would it be likely that the low number of predators observed caused the decline.

Finally, bell frog mortalities may have been due to disease and the presence of the Chytrid Fungus within the reintroduction site supports this as a cause of decline. The Chytrid Fungus causes the disease chytridiomycosis which can result in mass mortalities in amphibian populations and is thought to be the cause of the global amphibian decline that has been occurring for the past 30-40 years (Berger *et al* 1998). It currently infects at least 93 species around the world, 46 of which are Australian species (Speare and Berger 2004, 2005). The chytrid fungus has been found in bell frog populations within the Hunter Region (M.P. Stockwell unpubl. data) and although attempts were made to prevent it from entering the Hunter Wetlands Centre Australia reintroduction site through the exclusion of other frog species and the adoption of strict hygiene protocols, this was not successful.

The Chytrid Fungus was found on the skin of 53% of bell frogs surveyed in June 2005 with individuals showing an average count of 50 zoospores. This prevalence and zoospore count is much higher than those seen from free-living and non-declining bell frog populations (M.P. Stockwell unpubl. data) supporting the idea that chytrid infection was the cause of decline in the reintroduced population. Similarly, the discovery of dead bell frogs infected with chytrid suggests it was the cause of death, particularly as dead animals were found to have much higher levels of infection than live ones. However, this may be because the chytrid fungus continued to grow and multiply after the death of the frog and as no autopsies were performed on dead frogs, other causes of mortality cannot be ruled out. Despite this, the presence of infected animals in the reintroduction site, the knowledge that infection causes fatalities in bell frogs and is listed as a key threatening process of this species(DEC NSW 2005) makes death as a result of chytrid infection the most likely cause of decline in this reintroduced population.

The bell frog was not the only species to be infected with chytrid at this site but they were the only species observed to decline and not recover. The fungus was present in 3% of dwarf treefrogs sampled at the start of summer but because no bell frogs could be caught at that time the infection concentrations could not be compared between species. There are reports that the other two species, spotted treefrog and eastern froglet, can be infected by chytrid (Speare and Berger 2005) but this could not be confirmed by this study because too few animals were sampled to draw conclusions with a high level of confidence. The observation that only the bell frog declined in this study suggests that it may be more susceptible or more affected by chytrid infection than the other three species. These species may therefore be acting as reservoirs for the Chytrid Fungus, contributing to the bell frog decline. The observation of declines in the bell frog but not sympatric species in this study is echoed in nature suggesting these species may also be contributing to the decline of the bell frog throughout its range.

The finding that 2 dwarf treefrogs had low levels of chytrid and spotted treefrogs and eastern froglets can be infected also suggests that they may have carried the fungus into the site and swabbing all species more regularly may have confirmed this at an earlier stage of the reintroduction process. If these species did introduce chytrid into the reintroduction site, more effective fencing may also have prevented the population decline. Being carried by other frog species is not the only way that chytrid could have entered the site. A lapse in hygiene protocols may have resulted in chytrid being carried in on equipment or footwear. Recent studies have found that chytrid may be carried by other organisms such as on bird feathers (Johnson and Speare 2005) and reptile skin (Piotrowski *et al* 2004), both of which were able to move freely in and out of the reintroduction site and may have carried it in before or after the release of the bell frog. There is also some evidence that chytrid may persist in the environment in the absence of a host with laboratory studies showing chytrid can survive in lake water for up to 7 weeks (Johnson and Speare 2003). However, chytrid has never been isolated from the environment so it remains unknown how likely it was that chytrid was already present at the reintroduction site prior to the release of the bell frog.

The results of this study illustrate the importance of designing and monitoring programs to allow investigation into the causes of population fluctuations. For example, by monitoring habitat quality, predation, dispersal and disease it could be established that chytrid induced mortality was the most likely cause of decline in this reintroduced population, information that is essential for the success of future bell frog reintroduction programs. These results also provide the first evidence that the amphibian chytrid fungus can cause a rapid decline in bell frogs when a closed population containing one cohort is exposed to it. They show that an absence of sick and dead animals or the decline of sympatric species does not necessarily equate to an absence of the chytrid fungus, demonstrating the need for long term chytrid monitoring in both reintroduced and natural populations.

Finally, this study shows the importance of publishing the outcome of such reintroduction programs, whether they are successful or unsuccessful, because publishing allows reintroduction and monitoring methods to be refined, identifies problems, prevents mistakes from being repeated and promotes accountability in actions ensuring that well established reintroduction guidelines are followed (Jungius 1985; Dodd and Seigel 1991; Bloxam and Tonge 1995; IUCN/SSC 1995; Seddon *et al* 2007). It also allows us to see the effect that these programs have had on decreasing the species risk of extinction, and therefore their value in its conservation.

Acknowledgments

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